Table 8

Figure 19

Figure 19

Figure 19

4.7 Hemagglutination

Of the vaccinia virus strains used in the HA test, only CVA 2 and Elstree chicken erythrocytes were capable of agglutinizing (titer 1:128 and 1:32, respectively). The highly passaged strains MVA 574 , CVA 382 and CVA ens have lost this original ability. The constructs #MVA and #CVA 382 created by marker rescue of the host range gene were negative in the HA test.

4.8 Behavior in the incubator egg

As primary and secondary changes following infection of the chorioallantoin membrane with the initial strain CVA 2, large-scale foci developed with deep, central necrosis. Even in the case of high virus dilutions, substantial generalization occurred, along with damaging of the membrane vessels and subsequent death of the embryo.

In contrast, the primary changes following inoculation with the highly passaged virus strains MVA 574 and CVA 382 were characterized by small, white, compacted proliferation nodules without central necrosis. Although generalization involving tiny, proliferative secondary nodules distributed across the entire membrane occurred following an initial inoculation dose of 10^3 KID_{50}/0.1 ml, the embryos generally survived. The constructs #MVA and #CVA could not be differentiated from MVA 574 and CVA 382 in terms of their behavior.

Table 9

4.9 Behavior in the white mouse

All baby mice infected with the initial strain CVA 2 (i.c. or i.p.) died on the 2nd or 3rd day post-infection (p.i.). In contrast, all animals that were infected with MVA 574 or #MVA survived without exhibiting any signs off illness.

Signs of illness appeared on the 2nd day p.i. in adult mice infected intracerebrally with CVA 2. The animals were apathetic, their consumption of food and water was reduced, and their fur was matted. Beginning on the 3rd day p.i., additional central nervous system and respiratory symptoms appeared (ataxia, substantial increase in respiratory frequency, conjunctivitis). Three animals died between the 5th and 8th day p.i. Beginning on the 10th day p.i., the general condition of the remaining animals improved within one week, and the animals were completely healthy again 21 days p.i.

The mice that were given intraperitoneal infusions of CVA 2 exhibited a brief deterioration in their general condition on the 4th day p.i. Beginning on the 7th day p.i., easily visible, multiple pustules formed on the skin and on the tail. These pustules developed into wet, reddish and slightly swollen lesions (pox), which quickly scabbed over. Healing occurred quickly, and the wound scabs began dropping off 10-14 days p.i. During the formation of skin efflorescence,

the general condition of the animals remained undisturbed. There were no deaths.

Adult mice infected with MVA 574 and #MVA i.p. and i.c. survived. No signs of illness of any nature were observed.

Table 10

5. Discussion

Because of its unproblematic culturing and application, the vaccinia virus is an ideal vector virus for genetically engineered, recombined vaccines. Nevertheless, there are still substantial concerns in both human and veterinary medicine surrounding the use of vaccinia recombinants as vaccines:

1) Depending on the vaccinia virus strain, the residual virulence can lead to severe vaccine complications in high-risk patients.
2) What are the risks associated with recombination with other, naturally occurring orthopox species in the dissemination of a vaccinia vaccination virus produced by means of genetic engineering?

The only vaccinia virus strain that can be considered as a potential carrier for vaccine antigens is one that is well-characterized through markers and highly attenuated (Kaplan, 1989). For this reason, it is critical that the genetic basis of virus virulence be determined. One method of genetically determining virulence characteristics is the attenuation of virus strains by means of targeted deletion, insertion or point mutation of specific gene regions. As an alternative, the highly attenuated vaccinia virus strain MVA was compared, in this study, with its virulent initial strain CVA 2 and its 382nd HEF passage.

Our research shows that the initial material of the dermovaccinia strain Ankara presents itself as a heterogeneous virus population. Using cloning, we were able to isolate three plaque variants that are phenotypically identical but exhibit differences in the length of the terminal fragments. This is not uncommon, especially in the case of pox viruses. For example, Wittek et al. (1978b), in connection with their investigation of a commercially available vaccine population of the vaccinia virus strain Elstree, described a heterogeneity of the terminal fragments that could be eliminated by cloning. Other authors were unable to achieve similar results (Baroudy et al., 1982); they attribute this to the special conditions in the DNA replication of the pox viruses, during which the repeated and rapid duplication of repetitive sequences can occur at the genome end.

The total genome sizes we obtained, of 208,000 base pairs (bp) for CVA 2 and 200,000 bp for Elstree, lie at the upper limit of values cited in the literature for vaccinia viruses. However, they are based on the precise

molecular weight determination of large DNA fragments by means of sub-digestion with other restriction endonucleases. DNA fragments with high molecular weight, in particular, were previously estimated to be smaller following separation in conventional agarose gels (Mackett and Archard, 1979). Many studies, in this regard, appear to be oriented toward standard values determined only once. More recent studies (Bostock, 1988) use "pulsed field" gel electrophoresis to determine the genome size of the smallest mapped vaccinia virus strain, Western Reserve, at 192,000 base pairs (± 1 Kbp), thus confirming our results.

During the passaging of the vaccinia virus Ankara on HEF cultures, the genome size decreases from 208,000 base pairs (CVA 2) to 188,000 base pairs (CVA 382) to 177,000 base pairs (MVA 574). This corresponds to a loss of 15% of the entire genome. Mayr et al. (1978) only obtained an attenuation of approx. 9% for this strain. However, the total genome sizes they estimated (no data was provided on the size of individual DNA fragments) are, on the whole, significantly smaller than the values we obtained. 486 passages on embryonic pig kidney cells, which did not result in any attenuation of the virus, only affect the genome size to an insignificant extent. In comparison to the genome of CVA 2, only two deletions occurred, each occurring in the variable terminal fragments. Thus, the gene structure supports the slight attenuation tendency on embryonic pig kidney cells phenotypically described by Mayr et al. (1975).

The development of the physical gene cards was based on published data (Wittek, 1982). In particular, assignment was facilitated by the constant drifting behavior of many fragments following cutting with the restriction enzyme HindIII. In the gene cards for orthopox viruses developed by Mackett and Archard (1979), the occurrence of only one SmaI interface at the right end of the genome is viewed as typical for vaccinia viruses. Bostock (1988), however, discovered a second interface for the vaccinia virus Western Reserve at the left end of the genome. During the course of our studies, we were also able to localize a second SmaI interface in the genome of the vaccinia virus Elstree, in the XhoI A fragment, but only one SmaI interface in the CVA 2.

The results of the genome mapping clearly show that four deletions occurred during the course of the first 382 HEF passages. As Mayr et al. (1975) describe, the CVA initial virus lost its typical vaccinia characteristics during this passage period, especially with regard to virulence for rabbits, baby mice and chicken embryos. The phenotype of the vaccinia virus Ankara did not change as a result of 200 additional passages on HEF cells. Nevertheless, we were able to detect two additional deletions during our experiments.

A comparison of the physical cards of CVA 2, CVA 382 and MVA 574 shows that 2,900 base pairs were lost at the left end of the genome, directly at the transition between the ITR region and the "unique site" (Deletion I). Deletions in this genome region tend to occur after prolonged cell culture passaging (Moss et al., 1981b; Panicali et al., 1981; Paez et al., 1985). Apparently, this region is not essential for growth in cell cultures, although this deletion appears to be associated with a reduction in virulence (Buller et al., 1985; Dallo and Esteban, 1987). The genes of a number of secretory proteins were discovered in this segment (Kotwal and Moss, 1988a,b; Kotwal et al., 1989). A few of these "virokines," such as growth factor VGF (vaccinia growth factor) or a 35 KD polypeptide with complement binding characteristics, may interfere directly in the control mechanisms of the host organism, thereby influencing virulence. However, more detailed investigations of this region (such as marker rescue experiments), which appears to be significant with respect to virulence, have not yet been conducted. The extent to which these regions play a role in the attenuation of CVA 382 and MVA 574 also remains unanswered.

Deletion II has been investigated much more thoroughly. For the MVA virus, Altenburger et al. (1989) determined a deletion of 2,500 base pairs, which leads to the loss of more than two-thirds of the "host range" gene. Our investigations confirmed this scope of deletion. Consequently, the deleted region appears to cause the limited host range of CVA 382 and MVA 574. Gillard et al. (1986) discussed the extent to which the gene product of the "host range" gene, similar to the virokines mentioned above, co-determines virus virulence.

Deletion III is localized in the constant genome region in the right half of the genome, at the outermost right end of the HindIII A fragment. It compromises 4,200 base pairs and is characterized by the loss of a HindIII and an SmaI interface. Shida (1986) places the location of the hemagglutinin gene of vaccinia viruses in these genome region. For this reason, we investigated the hemagglutination (HA) behavior of various passages of vaccinia virus Ankara. While the initial strain CVA 2 exhibited a titer of 1:128 in the hemagglutination test, CVA 382 and MVA 574 were HA negative. It is possible that the hemagglutinin gene is completely or partially missing in CVA 382 and MVA 574. Flexner et al. (1987) demonstrated that a functional hemagglutinin gene verifiably contributes to an increase in virus virulence.

Deletion IV, the largest deletion determined (more than 10 Kbp), lies in the right end of the genome and encompasses a large portion of the ITR region. Similar deletions are known for a series of virus mutants in vaccinia, cowpox and monkeypox viruses (Moyer and Rothe, 1980; Archard et al., 1984; Esposito et al., 1981). The gene region, to the extent known, codes for phenotypic characteristics such pox morphology or host range. In this connection, Pickup et al. (1986) identified the gene for a protein that triggers the typical hemorrhagic changes in cowpox lesions. The gene of the growth factor (VGF) also lies in the ITR regions of both genome ends, and thus in the region of these deletions (Twardzik et al., 1985). Thus, the loss of more than 10,000 base pairs at the right end of the genome of vaccinia virus Ankara could also be responsible for the characteristic pox morphology on the chorioallantoin membrane of inoculated chicken eggs.

During the course of the passaging of CVA 382 to MVA 574, two additional deletions occur (totaling 10 Kbp), which, however, are not phenotypically remarkable. At the left end of the genome, similar to Deletion I, and in the center of the HindIII A fragment in the conserved region, 4,600 and 6,200 base pairs, respectively, are lost (Deletions V, VI). The only difference between CVA 382 and MVA 574 lies in their reaction with monoclonal antibodies (MAK). MVA 574 behaves like wild-type virus and host range mutants of the vaccinia virus Copenhagen (Sabine Johann, personal information). When the genomes of vaccinia virus Copenhagen and MVA 574 are compared,

it becomes apparent that the 4.6 Kbp Deletion V at the left terminal of MVA 574 is fully present in the 18 Kbp deletion of the host range mutants. Therefore, it can be assumed that the loss of 6,200 base pairs (Deletion VI) in the right half of the genome of MVA 574 is responsible for the lack of reaction with the MAK epitope. The applicable genome segment is among the best-conserved regions in the orthopox genome. In this context, Rodriguez and Esteban (1987) mapped the gene of a 14 KD polypeptide which, as a highly immunogenic, viral shell protein, plays an important role in the penetration of the host cell membrane.

During investigation of the protein structure, it was discovered, in the PAGE gel and immunoblot, that a 37 KD protein band of the initial strain CVA 2 attenuated to a 36 KD protein band of strains CVA 382 and MVA 574. Using MAK, Wilton et al. (1986) identified an immunogenic 35 HD shell protein in vaccinia virus, the locus of which could be localized in the HindIII H fragment (Gordon et al., 1988). In our investigation of the vaccinia virus Ankara, however, this gene region remained unchanged. The extent to which the protein we identified corresponds to the 35 KD polypeptide described, and/or which point mutations or micro-deletions resulted in the attenuation of the 37 KD protein band to CVA 2 remains unclear.

To summarize, it can be concluded that, on the basis of previously known gene functions, each of the Deletions I-IV could have influenced the attenuation of strains CVA 382 and MVA 574. The gene regions of Deletions I and II deserve special attention. In this segment, soluble proteins are coded that suggest previously unknown regulation mechanisms of virus replication in a host organism.

Three of the six regions we discovered to have deleted during the course of attenuation from CVA 2 to MVA 574 were suitable for the following tests:
1) Deletion II as a cause of the reduced host range of CVA 382 and MVA 574.
2) Deletion III as a presumed trigger of the HA negative phenotype.

3) Deletion VI, presumably responsible for the different reactions of CVA 382 and MVA 574 in the diagnostic Elisa.

For our subsequent investigations, we selected the host range gene region, because we wanted to know the extent to which the deletion in this region is solely responsible for the formation of the attenuated phenotype. In addition, the defect in the host range gene of the MVA virus had been precisely characterized in early studies (Altenburger et al., 1989). For this reason, the "host range" marker was well-suited as a selection system for marker rescue experiments.

The construct #MVA was created as a result of the targeted insertion of a 5.2 Kbp EcoRI fragment of CVA 2 into the genome of MVA 574. In theory, the selection of a subpopulation of MVA 574 that grows on the E-DERM cell line is also conceivable. However, this is contradicted by the fact that a construct #CVA 382 could be isolated when the same marker experiment was repeated with the strain CVA 382. Because, at the same time, multiple blind passages of CVA 382 and MVA 574 on E-DERM cells did not lead to virus isolation, and restriction enzyme analyses of the construct DNA revealed an intact host range gene region as the only difference, these initial concerns could be abandoned. The biological characteristics of the constructs #MVA and #CVA 382 are summarized and compared with those of CVA 2 and MVA 574 in Table 11.

The constructs #MVA and #CVA 382 behave, in almost all respects, in exactly the same manner as their initial strains MVA 574 and CVA 382. However, the host range is significantly expanded in comparison to these strains. Gillard et al. (1985; 1986) only tested the outcome of their marker rescue experiments for limiting the "human" host range gene on two cell lines, the mouse cell line CTK and the human cell line HEP 2. In contrast, the investigation of #MVA and #CVA 382 with 14 different cell lines showed that this host range gene not only facilitates replication ability on human cell lines, but also growth on animal cell lines, such E-DERM and RK 13. Nevertheless, the complete host range of the initial strain CVA 2 could not be reestablished solely by insertion of the host range gene region. This circumstance suggests that there are other gene regions in the genome of vaccinia viruses that characterize the cell range of the viruses.

The virus strain MVA 574, in contrast to the host mutant of vaccinia virus Copenhagen and despite its deletion in the host range gene, can still replicate on the human cell lines HEL (our own experiments) and 143 BTK (Altenburger et al., 1989). The extent to which the remainder of the host range gene has preserved residual activity in the genome of MVA 574, or whether other gene regions also play a role, has yet to be determined. In principle, however, the growth on different cell lines, controlled by the 32 KD polypeptide of the host range gene, appears more likely to be a factor of cell type and cell function than of the animal species from which the cell line is derived.

The constructs differ in terms of the cytopathic effect of initial strain CVA 2. They also develop only a slight tendency toward plaque formation on permissive cell lines, and the cytopathic effect of the constructs in the HEF culture remains very similar to MVA. The capacity to form plaque does not appear to be influenced by the insertion of the host range gene region. The only vaccinia gene known to affect the plaque phenotype to date – it codes a 14 KD shell protein – lies in the HindIII A fragment, in the center of the virus genome (Dallo et al., 1987).

Through in vitro translation and sequence analysis, it was possible to identify several polypeptides as gene products of the host range gene region of vaccinia viruses (Isle et al., 1981; Morgan and Roberts, 1984; Gillard et al., 1986). So far, however, no one has been able to determine the exact function of these proteins. Tamin et al. (1988) presumed that, as viral proteins, they interfere in the control of transcription in the nucleus of the host cell.

The constructs #MVA and #CVA 382 were investigated in PAGE gel, in the immunoblot and in the ELISA to clarify the protein structure. In this connection, #MVA behaved like MVA 574 and #CVA 382 like CVA 382. The insertion of the 5.2 Kbp EcoRI fragment of CVA 2 had no discernible effect on structure proteins or immunologically significant polypeptides of the constructs.

A relationship between host range and virulence has been assumed at various points in the literature (Mayr et al., 1978; Gillard et al., 1986). The reinsertion of the host range gene region into the gene of the well-characterized virus strains CVA 382 and MVA 574 was thus the first step toward testing this assumption. To this end, the constructs #MVA and #CVA 382 were tested in the cultured chicken egg and in the white mouse. While the virulence of CVA 2 was confirmed, in keeping with earlier studies (Mayr et al., 1975), #MVA and #CVA 382 proved to be just as avirulent as MVA 574. Apparently, the partial expansion of the host range is not associated with an increase in virulence. This conclusion is confirmed by the fact that the deletion of vaccinia genes, which have been proven to affect virulence, does not limit growth of the deletion mutants on various cell lines (Buller et al., 1988; Flexner et al., 1987). Kotwal et al. (1989) also describe a virus mutant with normal replication capacity in cell cultures, in which the deletion in the left half of the genome prevents the synthesis of soluble virus proteins, thereby weakening virulence. As the results of our genome mapping for the vaccinia virus Ankara demonstrate, these "virulence genes" lie in the region of the deletions we discovered, I, III and IV. However, they are unrelated to Deletion II in the host range region.

The biological characterization of the constructs #MVA and #CVA 382 prove, without a doubt, that the insertion of the host range gene regions of CVA 2 cannot reverse the avirulence of MVA 574 and CVA 382. A narrowed host range can therefore not automatically be viewed as an indicator of the

attenuation of a virus. However, the extent to which the degree of virulence is determined by the interaction among several "host range" genes with already known vaccinia genes that affect virulence remains unanswered. What also remains unanswered is the extent to which the point mutations or microdeletions not detectable in our experiments affect virulence. However, it appears reasonable, in further marker rescue experiments with constructs #MVA and #CVA 382, to gradually replace Deletions I, III and IV. Perhaps this will result in a better understanding of the complex relationships that lead to the development of virulence.

However, it can be assumed that the six mapped deletions are responsible for the attenuation of vaccinia virus Ankara. In this connection, the structure changes also affect several genome regions that are well-conserved in other orthopox viruses that have been studied. This favorable arrangement makes recombination of this classically attenuated (that is, through rapid passages in a heterologous host system) virus into a once again virulent wild type highly unlikely.

For this reason, the genome structure of the modified vaccinia virus Ankara justifies its use as a vaccine virus, whether in the conventional sense, that is, as a vaccine against orthopoxes, or – and this aspect seems to be considerably more significant today – as a safe carrier virus for foreign antigens.

[Translator's note: Summary is provided in German and English in the original document.]

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